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Research Article
Evaluation of the Antibacterial Potential of Ethanolic *Cannabis sativa* L. (Hang Kra Rog Phu Phan ST1) Extracts Against Human Pathogenic Bacteria

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Abstract

Background and Objective: Amid the escalating challenge of antibiotic resistance, the exploration of new sources has become essential, with plants serving as a promising reservoir of bioactive compounds. *Cannabis sativa* has attracted significant research interest for its antimicrobial properties and broad applications in medicine, industry and nutrition. This study aimed to investigate the antibacterial activity of ethanolic extracts from the stems and leaves of the Hang Kra Rog Phu Phan ST1 strain against twelve human pathogenic bacteria. **Materials and Methods:** Stems and leaves from the Hang Kra Rog Phu Phan ST1 strain were subjected to ethanol extraction. The primary antibacterial activity of ethanolic extracts from Tanao Si Kan Dang RD1 was assessed using the disc diffusion method, while the minimum inhibitory concentrations (MICs) and minimum bactericidal concentrations (MBCs) were determined via the broth microdilution method. The inhibition zone diameter (mm) was analyzed using Duncan's Multiple Range Test (DMRT) with the SAS software. **Results:** The findings revealed that the ethanolic extract from the leaves of Hang Kra Rog Phu Phan ST1 produced the largest inhibition zone diameter of 10.00 mm against *Bacillus subtilis* TISTR 008. The MIC and MBC of the leaf extract showed the lowest values of 0.09 and 0.19 mg/mL, respectively, recorded against *Staphylococcus aureus* TISTR 1466. **Conclusion:** This is the first report on the antibacterial activity of the ethanolic extracts from the leaves and stems of Hang Kra Rog Phu Phan ST1, which offers potential benefits for developing natural antibiotic drugs to combat infections caused by the tested pathogenic bacteria.

Key words: Anti-pathogenic bacterial activity, *Staphylococcus aureus* TISTR 1466, natural antibiotic drugs, Hang Kra Rog Phu Phan ST1 extracts

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Research has been conducted on cannabis due to its potential health benefits. These include managing pain[1], exhibiting anti-inflammatory effects[2], reducing nausea and vomiting[3,4], stimulating appetite, providing neuroprotection[5], antibacterial[6-8] and antifungal activities[9]. Additionally, it has been studied for its role in appetite loss[10], lowering blood pressure, treating cachexia and schizophrenia, managing anxiety disorders, fighting against cancer and HIV/AIDS and relieving gastrointestinal disorders[11].

In their 2008 study, Appendino *et al.*[12] reported on the antibacterial properties of cannabinoids derived from *Cannabis sativa*. The results demonstrated that all five major cannabinoids: Cannabidiol, cannabichromene, cannabigerol, Δ9-tetrahydrocannabinol and cannabinol-exhibited potent activity against a variety of strains of Methicillin-resistant *Staphylococcus aureus* (MRSA)[12]. The essential oils of *C. sativa* exhibited significant antibacterial activity, with a minimum inhibitory concentration (MIC) ranging from 0.5 to 32 µg/mL against *Staphylococcus aureus*, *Listeria monocytogenes*, *Enterococcus faecalis*, *Enterococcus hirae*, *Bacillus subtilis*, *Bacillus cereus* and *Bacillus* sp.[13]. Silver nanoparticles, synthesized using various *C. sativa* leaf extracts, were evaluated for their antibacterial properties. The results revealed that the combination of *C. sativa* leaf extracts and Silver Nanoparticles (AgNPs) inhibited the growth of *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens* and *Staphylococcus aureus*, with an inhibition zone diameter ranging from 7 to 14 mm[14]. Moreover, extracts derived from the leaves, seeds and stems of the *C. sativa* plant demonstrated antifungal activities against *Aspergillus oryzae*, *Aspergillus niger* and *Aspergillus parasiticus*[15]. Although research on the antibacterial properties of cannabis extracts against human-pathogenic bacteria is sparse, this study delved into the antibacterial potential of ethanolic extracts derived from the stems and leaves of Hang Kra Rog Phu Phan ST1, evaluated against twelve types of human-pathogenic bacteria. The findings underscore the prospective benefits of incorporating cannabis extracts into antibiotic formulations or as adjunctive therapy for patients suffering from infections triggered by the examined pathogenic bacteria.

MATERIALS AND METHODS

Study area: The research was conducted from July, 2023 to March, 2024 at the Faculty of Agricultural Innovation and Technology, Rajamangala University of Technology Isan, Nakhon Ratchasima and the Microbiology Laboratory within the Department of Science and Technology at the Faculty of Liberal Arts and Science, Roi Et Rajabhat University, Roi Et, Thailand.

Hang Kra Rog Phu Phan ST1 collection and preparation: Stems and leaves of the Hang Kra Rog Phu Phan ST1 were procured from the Rajamangala University of Technology Isan farm, located in Nakhon Ratchasima, Thailand (Fig. 1). The 1 kg of each collected plant part samples were thoroughly rinsed twice with tap water and subsequently segmented into smaller pieces. These samples were then subjected to a drying process in a hot air oven (POL-EKO-APARATURA, Wodzisław Śląski, Poland) at a temperature of 45°C for 3 days. The dried

Fig. 1: Hang Kra Rog Phu Phan ST1 at Rajamangala University of Technology Isan Farm

samples were then pulverized using an herb blender (WF-20B THAIGRINDER, Thailand). An extraction process was carried out on 70 g of the powdered samples using ethanol, involving a shaking period of 3 hrs. The resultant extract solution was collected via filtration and dried at a temperature of 50°C over 3 days. The final concentration of the stem and leaf extracts was adjusted to 500 mg/mL by the addition of Dimethyl Sulfoxide (DMSO, Sigma).

Human-pathogenic bacterial preparation: The study utilized five Gram-positive bacteria: *Bacillus cereus* TISTR 2373, *Bacillus subtilis* TISTR 008, *Enterococcus faecalis* 101, *Staphylococcus epidermidis* TISTR 518 and *Staphylococcus aureus* TISTR1466. Additionally, seven Gram-negative bacteria were also employed: *Escherichia coli* TISTR 780, *Escherichia coli* PK, *Escherichia coli* 101, *Klebsiella oxytoca* TISTR 556, *Klebsiella pneumoniae* TISTR 1838, *Pseudomonas aeruginosa* TISTR 2370 and *Pseudomonas aeruginosa* 101. These were selected due to their relevance as human pathogenic bacteria. Each bacterial strain was cultured from a single colony using nutrient broth (NB), under agitation at 37°C for 18 hrs. Before utilization, the bacterial concentration was standardized to an optical density (OD) of 0.1 at 600 nm[16-18].

Agar disc diffusion assay: The Agar disc diffusion assay was employed as the initial screening method for the antibacterial activity of the extracts. Inoculums of twelve strains of human-pathogenic bacteria were prepared afresh. Each strain of bacteria was cultured from an individual colony in 10 mL of nutrient broth (NB) and subjected to an incubation period overnight at a temperature of 37°C. Following incubation, the bacterial cells were centrifuged and the cell concentration was adjusted to an optical density (OD) of 0.1 at 600 nm. Subsequently, 100 µL of each bacterial suspension was aseptically spread onto nutrient agar (NA) plates. Sterile paper discs of 0.6 mm diameter were placed onto the NA plates. Each of these discs was impregnated with 10 µL of ethanolic extracts derived from the stems and leaves of Hang Kra Rog Phu Phan ST1, a procedure that was replicated thrice. The NA plates were then allowed a diffusion period of 15 min, followed by an incubation period of 24 hrs at 37°C in a bacterial incubator (Memmert, Willi-Memmert-Straße, Büchenbach). Post-incubation, the formation of inhibition zones around the paper discs was observed and recorded. The diameters of these zones, measured in millimeter (mm), served as indicators of bacterial susceptibility to the extracts, with larger zones signifying greater susceptibility.

MIC and MBC values assessment: The MIC and MBC serve as crucial parameters in the realm of antimicrobial susceptibility testing. The determination of MIC and MBC was carried out utilizing the micro broth dilution method, as previously delineated by Rattanasuk *et al*.[16]. The ethanolic extracts from the stems and leaves of Hang Kra Rog Phu Phan ST1 were serially diluted twofold in a 96-well culture plate containing NB, thereby achieving various extract concentrations (triplicated). Dimethyl Sulfoxide (DMSO) was employed as a negative control. Antibiotic-resistant bacteria, which demonstrated susceptibility in the antibacterial activity screening, were cultured using NB overnight. The bacterial concentration was meticulously adjusted to an optical density at 600 nm (OD600) of 0.1 before its addition to a 96-well culture plate. The plates were subsequently incubated in a bacterial incubator at 37°C overnight. Iodonitrotetrazolium chloride (INT), was used as an indicator dye. An aliquot of 50 µL of 2 mg/mL INT was added to each well, followed by an incubation period at 37°C for 30 min. The MIC value is defined as the lowest concentration of the extract that exhibits an inhibitory effect on the growth of the tested bacteria. Conversely, the MBC is the minimal concentration of the extract that results in the elimination of the tested bacteria, as evidenced by the absence of colour change post INT addition.

Data analysis: The inhibition zone diameter was analyzed using the SAS program version 5.0. The experiment followed a Completely Randomized Design (CRD) with three replicates per treatment, each consisting of five plates. Statistical analysis included Analysis of Variance (ANOVA) and mean comparisons across methods. A p-value of less than 0.05 was considered statistically significant. Duncan's Multiple Range Test (DMRT) was applied for mean comparisons.

RESULTS AND DISCUSSION

Antibacterial activity screening: The agar disc diffusion method is a commonly employed methodology for evaluating the susceptibility of bacterial strains to antimicrobial agents. This standardized protocol involves the strategic positioning of sterile paper discs, which have been saturated with predetermined concentrations of antimicrobial entities such as antibiotic medications and botanical extracts, onto an agar substrate that has been pre-inoculated with the intended pathogenic bacteria. The findings revealed that the ethanolic extracts of Hang Kra Rog Phu Phan ST1 leaves demonstrated an inhibition zone diameter ranging from

Table 1: Inhibition zone diameter of ethanolic Hang Kra Rog Phu Phan ST1 leaves extract against human pathogenic bacteria

Bacterial strain	Average of inhibition zone diameter (mm)*
Gram positive	
Bacillus cereus TISTR 2373	8^{bc}
Bacillus subtilis TISTR 008	10^{a}
Enterococcus faecalis 101	8^{bc}
Staphylococcus epidermidis TISTR 518	9^{ab}
Staphylococcus aureus TISTR1466	9^{ab}
Gram negative	
Escherichia coli TISTR 780	8^{bc}
Escherichia coli PK	8^{bc}
Escherichia coli 101	8^{bc}
Klebsiella oxytoca TISTR 556	8^{bc}
Klebsiella pneumoniae TISTR 1838	7^{c}
Pseudomonas aeruginosa TISTR 2370	8^{bc}
Pseudomonas aeruginosa 101	9^{ab}
p-value	0.0096

*Means (n = 3) in the column followed by the same common letter were not significantly different DMRT and p>0.05

Table 2: Inhibition zone diameter of ethanolic Hang Kra Rog Phu Phan ST1 stem extract against human pathogenic bacteria

Bacterial strain	Average of inhibition zone diameter (mm)*
Gram positive	
Bacillus cereus TISTR 2373	8
Bacillus subtilis TISTR 008	7
Enterococcus faecalis 101	7
Staphylococcus epidermidis TISTR 518	7
Staphylococcus aureus TISTR1466	8
Gram negative	
Escherichia coli TISTR 780	8
Escherichia coli PK	8
Escherichia coli 101	8
Klebsiella oxytoca TISTR 556	7
Klebsiella pneumonia TISTR 1838	7
Pseudomonas aeruginosa TISTR 2370	8
Pseudomonas aeruginosa 101	9
p-value	0.2408

*Means (n = 3) in the column followed by the same common letter were not significantly different DMRT and p>0.05

8 to 10 mm against Gram-positive bacteria, with the most substantial inhibition observed at 10 mm against *Bacillus subtilis* TISTR 008. Additionally, the inhibition zone diameter against Gram-negative bacteria ranged from 7 to 9 mm, with the maximum inhibition zone of 9 mm observed against *Pseudomonas aeruginosa* 101 (Table 1).

The results indicated that ethanolic extracts derived from Hang Kra Rog Phu Phan ST1 stem exhibited an inhibition zone diameter spanning 7 to 8 mm against Gram-positive bacteria. The most significant inhibition was observed at 8 mm against *Bacillus cereus* TISTR 2373 and *Staphylococcus aureus* TISTR 1466. Moreover, the inhibition zone diameter against Gram-negative bacteria ranged from 7 to 9 mm, with the highest inhibition zone measuring 9 mm against *Pseudomonas aeruginosa* 101 (Table 2).

Minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC): The determination of the MIC and MBC was conducted using the micro broth dilution method. The extracts from the stems and leaves of Hang Kra Rog Phu Phan ST1 were subjected to a two-fold dilution, followed by the addition of the bacteria under test. A colorimetric assay was employed to quantify the antibacterial activity. The MIC values of the ethanolic extracts from Hang Kra Rog Phu Phan ST1 leaves demonstrated that the lowest MIC value of 0.09 mg/mL was achieved against *Staphylococcus aureus* TISTR1466. This was followed by a MIC value of 3.12 mg/mL against *Bacillus subtilis* TISTR 008 and *Staphylococcus epidermidis* TISTR 518 for Gram-positive bacteria. For Gram-negative bacteria, the lowest MIC value of 3.12 mg/mL was observed against *Escherichia coli* TISTR 780 and *Escherichia coli* PK. Subsequently, an MIC value of 6.25 mg/mL was noted against *Escherichia coli* 101, *Klebsiella oxytoca* TISTR 556, *Pseudomonas aeruginosa* TISTR 2370 and *Pseudomonas aeruginosa* 101.

The MBC values of the ethanolic extracts from Hang Kra Rog Phu Phan ST1 leaves demonstrated that the lowest MBC value of 0.19 mg/mL was presented in *Staphylococcus aureus*

Table 3: MIC and MBC values of ethanolic Hang Kra Rog Phu Phan ST1 leaves extract against human pathogenic bacteria

Bacterial strain	MIC (mg/mL)	MBC (mg/mL)
Gram positive		
Bacillus cereus TISTR 2373	6.25	12.50
Bacillus subtilis TISTR 008	3.12	12.50
Enterococcus faecalis 101	6.25	12.50
Staphylococcus epidermidis TISTR 518	3.12	25.00
Staphylococcus aureus TISTR1466	0.09	0.19
Gram negative		
Escherichia coli TISTR 780	3.12	12.50
Escherichia coli PK	3.12	12.50
Escherichia coli 101	6.25	12.50
Klebsiella oxytoca TISTR 556	6.25	25.00
Klebsiella pneumoniae TISTR 1838	12.50	25.00
Pseudomonas aeruginosa TISTR 2370	6.25	12.50
Pseudomonas aeruginosa 101	6.25	12.50

MIC: Minimum inhibitory concentration and MBC: Minimum bactericidal concentration

Table 4: MIC and MBC values of ethanolic Hang Kra Rog Phu Phan ST1 stem extract against human pathogenic bacteria

Bacterial strain	MIC (mg/mL)	MBC (mg/mL)
Gram positive		
Bacillus cereus TISTR 2373	6.25	12.50
Bacillus subtilis TISTR 008	6.25	12.50
Enterococcus faecalis 101	12.5	25.00
Staphylococcus epidermidis TISTR 518	6.25	25.00
Staphylococcus aureus TISTR1466	6.25	25.00
Gram negative		
Escherichia coli TISTR 780	3.12	12.50
Escherichia coli PK	3.12	12.50
Escherichia coli 101	6.25	12.50
Klebsiella oxytoca TISTR 556	12.5	25.00
Klebsiella pneumoniae TISTR 1838	12.50	25.00
Pseudomonas aeruginosa TISTR 2370	6.25	12.50
Pseudomonas aeruginosa 101	6.25	12.50

MIC: Minimum inhibitory concentration and MBC: Minimum bactericidal concentration

TISTR1466. Followed by a MIC value of 12.5 mg/mL against *Bacillus cereus* TISTR 2373, *Bacillus subtilis* TISTR 008 and *Enterococcus faecalis* 101 for Gram-positive bacteria. In the case of Gram-negative bacteria, the lowest MBC value of 12.50 mg/mL was obtained from *Escherichia coli* TISTR 780, *Escherichia coli* PK, *Escherichia coli* 101, *Pseudomonas aeruginosa* TISTR 2370 and *Pseudomonas aeruginosa* 101 (Table 3).

The MIC values of the ethanolic extracts from Hang Kra Rog Phu Phan ST1 stems against Gram-positive bacteria revealed the lowest MIC value of 6.25 mg/mL against *Bacillus cereus* TISTR 2373, *Bacillus subtilis* TISTR 008, *Staphylococcus epidermidis* TISTR 518 and *Staphylococcus aureus* TISTR1466. This was followed by an MIC value of 12.5 mg/mL against *Enterococcus faecalis* 101. In the case of Gram-negative bacteria, the lowest MIC value of 3.12 mg/mL was noted against *Escherichia coli* TISTR 780 and *Escherichia coli* PK, followed by 6.25 mg/mL against *Escherichia coli* 101, *Pseudomonas aeruginosa* TISTR 2370 and *Pseudomonas aeruginosa* 101.

The MBC values of the ethanolic extracts from Hang Kra Rog Phu Phan ST1 stems against Gram-positive bacteria showed that the lowest MBC value of 12.5 mg/mL was observed against *Bacillus cereus* TISTR 2373, *Bacillus subtilis* TISTR 008, *Staphylococcus epidermidis* TISTR 518 and *Staphylococcus aureus* TISTR 1466. This was followed by an MBC value of 25.00 mg/mL against *Enterococcus faecalis* 101, *Staphylococcus epidermidis* TISTR 518 and *Staphylococcus aureus* TISTR 1466. For Gram-negative bacteria, the lowest MBC value of 12.5 mg/mL was observed against *Escherichia coli* TISTR 780, *Escherichia coli* PK, *Escherichia coli* 101, *Pseudomonas aeruginosa* TISTR 2370 and *Pseudomonas aeruginosa* 101. This was followed by an MBC value of 25.00 mg/mL against *Klebsiella oxytoca* TISTR 556 and *Klebsiella pneumoniae* TISTR 1838 (Table 4).

The outcomes of this study, which examined the antibacterial activity of the ethanolic extract from the leaves and stems of Hang Kra Rog Phu Phan ST1, are consistent with prior research by Serventi *et al.*[19]. They highlighted the antibacterial potency of the hydroalcoholic extract from

C. sativa L. cv. *strawberry* inflorescences[11-15]. The findings showed that the extracts had minimum inhibitory concentration (MIC) values between 4.96 and 39.68 µg/mL against *Escherichia coli*, 1.56 to 19.8 µg/mL against *Bacillus subtilis*, 39.68 to 62.99 µg/mL against *Pseudomonas aeruginosa* and 15.74 to 62.99 µg/mL against *Staphylococcus aureus*. This aligned with the work of Appendino *et al*.[12], who reported that cannabinoids from *C. sativa* inhibited the growth of *Staphylococcus aureus* with MIC values ranging from 0.125 to 128 µg/mL. Furthermore, Iseppi *et al*.[13] reported on the antibacterial activity of essential oils from fibre-type *Cannabis sativa* L. against bacterial pathogens. Their results showed MIC values ranging from 2-32 µg/mL against *Staphylococcus aureus*, 1-16 µg/mL against *Staphylococcus epidermidis*, 0.5-32 µg/mL against *Enterococcus faecalis*, 1-26 µg/mL against *Bacillus subtilis* and 1-16 µg/mL against *Bacillus cereus*. Csakvari *et al*.[14] conducted a study on the antibacterial properties of silver nanoparticles synthesized by mixing various *C. sativa* leaf extracts. Their findings demonstrated that the combination of silver nanoparticles and *C. sativa* leaf extracts exhibited effective antibacterial activity against *Escherichia coli*, *Klebsiella pneumoniae*, *Pseudomonas fluorescens* and *Staphylococcus aureus*. These findings suggested that the ethanolic extract from the leaves and stems of Hang Kra Rog Phu Phan ST1 could inhibit or reduce the proliferation of human-pathogenic bacteria, offering potential benefits for the development of natural antibiotic drugs.

CONCLUSION

This study represents the inaugural investigation into the efficacy of ethanolic extracts derived from the leaves and stems of Hang Kra Rog Phu Phan ST1 in combatting human pathogenic bacteria. The findings reveal that these extracts exhibit potent antibacterial activity against a spectrum of pathogens, including *Bacillus cereus* TISTR 2373, *Bacillus subtilis* TISTR 008, *Enterococcus faecalis* 101, *Staphylococcus epidermidis* TISTR 518, *Staphylococcus aureus* TISTR 1466, *Escherichia coli* TISTR 780, *Escherichia coli* PK, *Escherichia coli* 101, *Klebsiella oxytoca* TISTR 556, *Klebsiella pneumoniae* TISTR 1838 and *Pseudomonas aeruginosa* TISTR 2370. Consequently, these extracts from Hang Kra Rog Phu Phan ST1 hold significant promise as potential therapeutic agents for treating infections caused by the tested bacterial strains.

SIGNIFICANCE STATEMENT

This study investigates the antibacterial activity of ethanolic extracts from Hang Kra Rog Phu Phan ST1 against twelve human pathogenic bacteria. The findings of this research demonstrate the antibacterial activity of ethanolic extracts from the leaves and stems of Hang Kra Rog Phu Phan ST1. These extracts effectively eliminate the tested human pathogenic bacteria. By exploring previously uncharted territory, this research sheds light on the potential use of ethanolic Hang Kra Rog Phu Phan ST1 extracts as a natural antibiotic. Consequently, novel applications leveraging their antibacterial properties may emerge. Future studies should focus on isolating and identifying the active compounds within Hang Kra Rog Phu Phan ST1 extracts, as well as conducting clinical trials to assess their safety and efficacy as natural antibiotics for potential therapeutic use.

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